The entitled thesis describes our studies on “Haematological and Bio-Chemical studies of fish exposed to Cadmium salts”. The metal pollutants are one of those having longest life and, therefore, can cause impact on the ecological systems for a prolonged period of time. This has been actually the inspiration to undertake the aforementioned studies.

Introduction

Water is the most abundant of all compounds on earth, and is essential for the continued existence of life on this planet. It covers approximately 71% of the planet and is present in all living matter, at times comprising as much as 90% of the body’s tissues. Of all available water on earth, only 0.25% can be classified as surface water (Davis et al. 1998). Over the years there has been an increased interest in the effects of human habitation and industrialization on water resources. With the earth’s population increasing steadily every year, more pressures are being placed on this precious resource.

The aquatic environments are constantly being exposed to pollution from various sectors. These include industrial processes, mining operations, and agricultural and domestic run-off (Förstner et al. 1979).

Main sources of water pollution are:

- Sewage and other wastes (Resulting in Eutrophication)
- Industrial Effluents (Oils, Grease, Plastic, Metallic wastes like Cu, Cd, Cr, Hg, Pb etc, Toxins, Acids etc.)
- Agricultural Discharges (Fertilisers, Pesticides, Biocides etc.)
- Industrial Wastes(Physical pollutants, Heat, Radioactive waste)

One of the sources of pollution is from heavy metals, which regularly find their way into the aquatic ecosystem. Geological weathering, industrial processing of metals and ores, the use of metal compounds, leaching of metals from garbage and solid waste dumps, and animal and human excretions that may contain heavy metals have all contributed to the rise of metals in these fragile

ecosystems (Förstner et al. 1979). Being elements, metals cannot be broken down or destroyed by degradation, but instead accumulate within the environment in different forms.

Metallic elements are intrinsic components of environment and are difficult to be removed completely from environment. The impact of these toxic agents on human health is currently an area of intensive research, due to the ubiquity of exposure. With increasing use of wide variety of metals in industries and our daily life, problems arising from toxic metal pollution of the environment have assumed serious dimensions.

Ecotoxicology

Ecotoxicology in its broader definition is a science that deals with the fate and the impact of substances on the environment. Ecotoxicological effects mean the interference of substances with living organisms. Exposure to harmful substances can cause acute or chronic effects in organisms. The effect may be directed at, for example, the organism's genome or vital biochemical functions. The possible environmental fates and effects of chemicals and environmental samples are often studied in laboratory-scale.

Ecotoxicological assessment provides tools and data for environmental risk assessment (ERA). ERA is an integral part of environmental impact assessment and it can be used to predict possible ecological risks of, for example, wastewaters. Ecotoxicological data is used to calculate predicted environmental concentrations (PEC) and predicted no-effect concentrations (PNEC), which are key parameters of ERA. Laboratory tests using the contaminated matrix or biomarker studies from exposed organisms can be used for assessing the significance of pollution and the required remedial processes.

Although risk assessments of pure chemicals and environmental samples do differ, the ecotoxicological test set-ups may be rather similar. However, when concerning methods for environmental samples, especially the possible effects of the matrix and sampling conditions have to be carefully considered. Chemical risk assessments are usually carried out on single substances, although in the environment they are functioning as parts of compound mixtures.

In the past, chemical analysis of water has proven to be of great use for the detection of pollutants within the environment. But the reliance on chemical data alone is becoming obsolete. There is now a trend to incorporate biological monitoring, or biomonitoring, into standard water analysis.

Biomonitoring allows for the analysis of the effect of pollution on biological systems, providing an indication of both the past and the present water quality. Additionally, biomonitoring at the lower levels of biological organisation allows for an "early warning system" whereby stressors can be detected at an early stage, and dealt with before they exert their effects in higher levels of biological organisation, e.g. at individual or community level. Fish are the ideal models for addressing questions regarding pollution in the aquatic environment, as they are naturally exposed to a complex selection of environmental perturbations. When fish are subjected to stressors such as water pollution, they have immediate neuro-endocrine changes (regarded as the primary response) that produce secondary biochemical and physiological changes. These changes ultimately lead to changes at the individual, community or population level (Pickering et al.1995).

Thus, eco-toxicological effect studies are usually carried out by exposing a biological receptor for a series of sample dilutions. Effects of these diluted samples are compared to an unexposed set-up. As a result, usually EC-values and/or LOEC and NOEC values are calculated depending on the test set up and purpose. EC_{50} value (effective concentration value) represents the concentration of the studied sample that causes an effect on half (50%) of the tested system. The lowest observed effective concentration (LOEC) is the test concentration one step higher from the NOEC-value. Hence, the selected test concentrations with the factor used between the successive concentrations are of major importance.

Different endpoints can be used for the quantification of harmful effect. Reproduction, growth and survival are endpoints that have been traditionally used in whole organism tests. Many of these types of responses can be detected by eye and the test durations are quite long, especially if they are to cover the organism's full life cycle. On the other hand, methods based on

changes in certain cellular or sub-cellular reactions and activities, as well as on microbes as test organisms, have started to be used more and more. These assays are popular for their small-size and short test duration but need special instrumentation to detect the response. Development of such new test methods has been driven by the wish to diminish the use of vertebrate tests.

A wide variety of rapid small-scale tests have been developed as possible alternatives for the traditional tests. In addition to the possibility to reduce the use of vertebrates, small-scale tests are also quite fast and (semi-)automation of the procedures is often possible. Thus, besides the ethical considerations, small-scale methods are also economically more feasible and thus more potential for routine control and assessment use. They are often relatively sensitive and thus also reduce the risk of the biological effects being underestimated. Results from small scale tests are often compared to traditional whole organism tests. But, it is not yet clear how well small-scale tests resemble the responses in whole organisms in actual environmental conditions.

Motivation

India is a country blessed with ecological and mineral diversity surpassing most other countries in the world. However, this does have its drawbacks. The release of metals into the environment as a result of the rapid industrialization activities, especially in Industrial belt from Ahmedabad to Bombay may have serious implications on the biota. More specifically, the increased global usage of Cadmium and its products is of great concern, as increases in Cadmium are a known cause of physiological damage in many organisms.

The ability to monitor and predict the effects of metals on organismal processes at the various levels of biological organisation may be considered greatly advantageous to the continued survival of a species, and beneficial to sustaining the state of the aquatic ecosystem. Identification of problems originating at the lower levels of biological organisation (for example alterations of functionality of various organs, stress protein accumulations or histological changes) will prove to be valuable tools in the assessment of possible insults on environments.

Motivation of the study is as follows:

- The effects of alterations in the sub-cellular protective components of the cells may have an effect on higher levels of biological organisation, e.g. at tissue level, and needs to be investigated further. Hence the purpose of histological studies.
- The effect of the type of acclimatization medium on biomarker response needs to be identified.
- There exists limited amount of information regarding haematological effects of Cadmium exposure on fish under investigation.
- There exists limited amount of information regarding effect of Cadmium exposure on functionality of liver and kidney of the fish under investigation.

Objectives of the Study

The Objective of this study is as follows:

- To identify what are the effects of Cadmium exposure on the fish under investigation.
- To estimate the acute toxicity data (namely LC_{50}, NOEC, Upper and Lower confidence level)
- To estimate the Chronic toxicity data (Haematological effects, biochemical effects on fish)

Description of the Research work

For the experiment purpose *Oriochromis niloticus* was selected as the animal to be studied. Fish were collected from a local fish farm in the village Napad, Dist. Anand and allowed to acclimatize with the laboratory condition for 2 weeks in a large tank, keeping temperature, hardness, and pH in controlled condition. The fish were fed 3 times a day.

For the acute toxicity measurements the fish were divided into 6 groups each of 10 and exposed to different concentrations of $CdCl_2$. The exposure period was

of 96 hours. The same experiment was repeated twice and with the collected data, acute toxicity results were achieved.

The acute toxicity results are as follows:

concentration (in mg/l)	Exposure Time (in Hours)									
	1	2	4	6	12	24	36	48	72	96
Control (0mg/l)	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	1	1	2	2
3	0	0	0	0	0	0	1	2	3	4
4	0	0	0	0	1	1	2	4	5	6
5	0	0	0	1	1	2	3	4	6	7

LC_{50} Value=3.5603mg/l

concentration (in mg/l)	Exposure Time (in Hours)									
	1	2	4	6	12	24	36	48	72	96
Control (0 mg/l)	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	1	1	1
3	0	0	0	0	0	0	1	2	3	3
4	0	0	0	0	1	2	3	5	6	7
5	0	0	0	1	1	2	4	5	8	9

LC_{50} Value=3.5174mg/l

Further, in order to estimate the chronic effects, again the fish were divided into 4 groups each having 30 fish. The fish were exposed to different $CdCl_2$ concentrations for 15 days. After exposure of 24 hours, 7 days and 15 days fishes were sacrificed to collect blood samples from them. The collected blood samples were analyzed for measurement of different haematological parameters like Hb, RBCs, Haematocrit (Hct), MCH, MCHC and biochemical parameters like Creatinine, SGPT, triglyceride, Alkaline Phosphatase, Glucose, Cholesterol, Total Protein and Aspartate amino transferase.

For blood analysis, the caudal peduncle was cut; blood was collected in heparinized tubes. Total red blood cells count (RBC) was determined according to the classical method using the Neubauer haemocytometer. Haemoglobin

content (Hb) was estimated using Sahli (acid haematin) method. Haematocrit value (Hct) was measured using microcapillary reader; Mean Cell Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were estimated using the following expressions.

MCHC (%) = (Hb / Hct) x 100
MCH (pg) = (Hb / RBC) x 10
MCV (μ^3) = (Hct / RBC) x 10

For Glucose analysis Trinders method, for Triglyceride GPO–POD Enzymetic colorimetry, for Creatinine Jaffe colorimetric-kinetic, for Cholesterol CHOD–PAP End point, for SGOT (AST) IFCC method, Kinetic without pyridoxal method were employed.

Results obtained from the chronic tests are summarized as follows:

Haematological Studies

		Hb(gm/dl)		Hct(%)		MCV(μm^3)		MCH (pg/cell)		MCHC (gm/dl)		RBC ($10^6/mm^3$)	
24 Hr	Control	5.70	± 0.10	35.26	± 1.07	191.19	± 11.34	30.91	± 1.63	16.17	± 0.42	1.85	± 0.07
	0.1	5.33	± 0.06	33.33	± 1.03	201.80	± 10.54	32.28	± 1.32	16.01	± 0.50	1.65	± 0.05
	0.2	5.23	± 0.06	31.40	± 1.30	198.32	± 15.59	33.04	± 1.85	16.68	± 0.56	1.59	± 0.07
	0.3	4.90	± 0.10	30.56	± 1.32	204.16	± 21.41	32.70	± 2.62	16.04	± 0.38	1.50	± 0.09
7 Days	Control	5.47	± 0.12	33.50	± 1.03	198.61	± 25.16	32.33	± 2.86	16.33	± 0.81	1.70	± 0.16
	0.1	4.63	± 0.15	30.44	± 0.79	209.53	± 6.50	31.93	± 2.12	15.24	± 0.87	1.45	± 0.05
	0.2	4.43	± 0.06	29.02	± 1.36	207.87	± 7.65	31.79	± 1.78	15.30	± 0.87	1.40	± 0.06
	0.3	4.10	± 0.10	26.71	± 1.51	203.51	± 17.65	31.29	± 3.07	15.38	± 0.83	1.32	± 0.09
15 Days	Control	5.10	± 0.10	31.55	± 0.63	188.43	± 10.02	30.46	± 1.78	16.17	± 0.33	1.68	± 0.07
	0.1	4.07	± 0.15	27.40	± 1.03	207.72	± 18.80	30.78	± 1.81	14.86	± 0.97	1.32	± 0.07
	0.2	3.60	± 0.10	26.31	± 1.10	223.26	± 15.51	30.54	± 1.67	13.69	± 0.20	1.18	± 0.04
	0.3	3.33	± 0.15	25.16	± 0.19	225.59	± 11.02	29.90	± 2.22	13.25	± 0.56	1.12	± 0.05

Results obtained from the Bio-chemical tests are summarized as follows:

Bio-chemical Studies

		Creatinine (mg/dl)		Total prt (gm/dl)		TriGlyceride (mg/dl)		Glucose (mg/100ml)		Cholesterol (mg/dl)	
24 Hr	Control	0.14	± 0.01	3.82	± 0.44	404.13	± 3.58	81.92	± 1.20	105.93	± 1.10
	0.1	0.17	± 0.01	3.78	± 0.46	408.47	± 3.00	86.97	± 0.92	111.17	± 1.67
	0.2	0.21	± 0.01	3.69	± 0.44	411.80	± 1.56	93.20	± 1.73	115.50	± 1.73
	0.3	0.23	± 0.01	3.56	± 0.48	413.73	± 1.10	112..57	± 2.37	121.73	± 1.85
7 Days	Control	0.18	± 0.03	3.86	± 0.46	412.00	± 4.33	83.15	± 1.00	112.73	± 4.04
	0.1	0.24	± 0.01	3.27	± 0.22	422.20	± 0.69	121.00	± 3.12	121.97	± 1.15
	0.2	0.29	± 0.01	3.11	± 0.12	427.20	± 1.91	134..97	± 4.39	129.53	± 2.48

	0.3	0.35	± 0.01	2.91	± 0.06	436.80	± 1.91	141..63	± 4.27	135.70	± 2.60
15 Days	Control	0.29	± 0.12	3.90	± 0.44	409.13	± 0.75	82.81	± 1.81	109.50	± 6.41
	0.1	0.32	± 0.01	3.03	± 0.24	436.40	± 2.25	152..27	± 4.91	140.73	± 0.81
	0.2	0.37	± 0.01	2.88	± 0.30	443.80	± 5.37	162..53	± 2.89	156.40	± 3.98
	0.3	0.44	± 0.01	2.76	± 0.30	447.47	± 2.19	170..97	± 2.31	169.97	± 0.75

		Ast (U/L)		SGPT (IU/L)		Alkaline Phosphatase (IU/L)	
24 Hr	Control	41.89	± 0.57	27.13	± 1.85	48.30	± 0.12
	0.1	56.24	± 1.76	31.20	± 0.69	48.10	± 0.75
	0.2	67.21	± 1.06	30.73	± 1.10	47.50	± 0.58
	0.3	81.72	± 2.20	35.13	± 0.75	47.10	± 0.58
7 Days	Control	53.59	± 1.60	31.80	± 1.04	47.90	± 1.10
	0.1	74.64	± 1.58	38.80	± 0.69	46.30	± 0.64
	0.2	86.82	± 1.47	42.03	± 0.40	45.20	± 0.64
	0.3	94.46	± 1.27	46.00	± 0.00	45.10	± 1.15
15 Days	Control	65.12	± 1.20	35.27	± 0.64	48.10	± 0.64
	0.1	92.56	± 1.19	41.57	± 0.81	45.10	± 0.64
	0.2	103.99	± 1.11	43.70	± 0.87	43.90	± 0.17
	0.3	109.17	± 0.96	48.77	± 0.46	41.80	± 0.40

Conclusion

Cadmium severely impairs various haematological parameters and poses a threat to the biological functions of fish.

Cadmium causes a decrease of blood iron level which may be the cause of decreased concentration of haemoglobin. Cadmium caused a reduction in erythropoisis and impeded the formation of the RBCs. Cadmium induced anaemia appears to be associated with a defect in iron metabolism caused by a deficiency in intestinal absorption. The anaemic condition in fish results from an unusually low number of red blood cells or too little haemoglobin in the red blood cells.

Haemoglobin is the oxygen-carrying component in the blood of fish and its concentration can be used as a good indicator of anaemia (Blaxhall et al. 1973). The reduction in haemoglobin content in fish exposed to toxicant could also be due to the inhibitory effect of the toxic substance on the enzyme system responsible for synthesis of haemoglobin (Pamila et al.1991). Cadmium might alter the properties of haemoglobin by decreasing their affinity towards oxygen binding capacity rendering the erythrocytes more fragile and permeable, which probably results in cell swelling, deformation and damage (Witeska et al. 2003). Decreased haemoglobin following metal exposure usually results in haemodilution. Haemodilution (Adeyemo 2005 and Ayuba 2008) resulting from

impaired osmoregulation across the gill epithelium and it could be as a result of the destruction of intestinal cells. This haemodilution has been regarded as a mechanism that reduces the concentration of the toxicant pollutant in the circulatory system (Srnit et al., 1979).

Decreases in the red blood cells could also be as a result of internal bleeding caused by damaged kidney. Erythropaenia could also be as a result of damaged gills and impaired osmoregulation during sublethal cadmium exposure, which cause haemodilution leading to a decrease in the number of red blood cells through haemolysis (Wedemeyer et al.1977).

Haematocrit is used to determine the ratio of plasma to corpuscles in the blood as well as the oxygen-carrying capacity of the blood (Larsson et al., 1985). The significant decrease in the haematocrit in this study could be attributed to gill damage and/or impaired osmoregulation causing anaemia and haemodilution. The observed reduction in the haemoglobin and haematocrit values in the fish could also be attributed to the lysing of erythrocytes.

The reduction in RBCs, Haematocrit and Haemoglobin in the fish upon exposure to sublethal levels of cadmium might be due to the destruction of mature RBCs and the inhibition of erythrocyte production due to reduction of haemosynthesis that affected by pollutants, (James et al. 1999). The significant reduction in the same parameters are reported to be indication of severe anaemia caused by destruction of erythrocytes (Omoniyi et al. 2002,; Kori-Siakpere et al. 2009).

Unlike MCHC values, the Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Volume (MCV) increased with increased concentrations of toxicant. The significant change in the MCH may be due to the reduction in cellular blood iron, resulting in reduced oxygen carrying capacity of blood and eventually stimulating erythropoiesis (Hodson et al.1978). The related decrease in haematological indices proved the toxic effect of heavy metals that affect both metabolic and haemopoietic activities.

Environmental stressors such as metal exposure (Yang et al. 2003; Levesque et al. 2002; Zikic et al. 2001) may change the biochemical parameters. Therefore the measurement of serum biochemical parameters can be useful as diagnostic

tool in toxicology to find their general health status and target organs affected by toxicants (Zikic et al, 2001; Mc Donald et al. 2006).

The present study demonstrated that the *Oriochromis niloticus* exposed to sublethal concentrations of cadmium displayed a significant elevation in the level of blood glucose after all the exposure periods. Similar observations have been reported by Benson et al. (1987), Early et al. (1990), Partap et al. (1990), Hontela et al. (1996), in fish and rat treated with cadmium. High levels of blood glucose are caused by disorders in carbohydrate metabolism appearing in the condition of physical and chemical stresses. A variety of stressors stimulate the adrenal tissue resulting in increased level of circulating glucocorticoids and catecholamines. Both of these produce hyperglycaemic condition. Hyperglycaemic response in this study is an indication of a disrupted carbohydrate metabolism possibly due to enhanced breakdown of liver glycogen. Wedemeyer et al. (1981) reported that the elevation in the blood glucose level is response to the increased rate of glycogenolysis or gluconeogenesis (Sastry et al. 1985).

The observed decrease of plasma protein could also result from the breakdown of protein into amino acids first and possibly into nitrogen and other elementary molecules. When exposed to stressors, the gills become “leaky” to water and ions, often resulting in osmoregulatory imbalances. Therefore, the decline in serum total protein might have been attributed to several pathological processes including plasma dissolution, renal damage and protein elimination in the urine, a decrease in liver protein synthesis, alteration in hepatic blood flow and/ or hemorrhage into the peritoneal cavity and intestine (Salah El-Deen et al., 1996). The decrease in plasma and tissue protein may occur due to the increase of protein breakdown as a result of stimulated corticosteroid hormones which to provide amino acids for enhance the breakdown of proteins and gluconeogenesis to provide glucose to compensate for increase in energy demands under stressful condition.

Cholesterol concentrations in the serum of metal-exposed fish generally increased when compared to the control value (Muazzez et al., 2009). The concentrations of cholesterol is an essential structural components of

membranes and the precursor of all steroid hormones, may increase due to the liver failure causing the release of cholesterol into the blood. Hyper cholestrolemia observed may be due to impairment of liver and inhibition of enzymes, which converts cholesterol into bile acid (Murrey 1990). Reduced lipoprotein lipase activity plays a role in the increment of plasma lipid (Asha Agrawal et al. 1999). The increased levels of cholesterol develop weakness in the body and swimming ability of the fish was observed to decrease in our study.

Several reporters showed that these blood enzymes such as SGPT(Serum Glutamate Pyruvate Transaminase) and AST (Aspartate amino Transferase) were highly increased in the fish treated with cadmium, zinc and copper (Benson et al. (1987); Atef (2005); Singh et al. (1990); Hilmy et al. (1987); Karan et al., (1998). The increase in blood enzymatic activity is either due to (i) leakage of these enzymes from hepatic cells and thus raising levels in blood, (ii) increased synthesis and (iii) enzyme induction of these enzymes. Tietz (1987) and Campbell (1984) reported that these enzymes liberate to the blood stream when the hepatic parenchyma cells are damaged. The increased serum transaminase (AST) may reflect hepatic toxicity which leads to extensive liberation of the enzymes into the blood circulation (Daabees et al., 1992). The tissue damaged by toxicants exhibit a sharp rise in activity of mitochondrial enzymes Aspartate amino transferase (Abdelsalam et.al. 1982, Mikhail et.al. 1979). Aspartate amino transferase has been strongly implicated in the production of energy in tissues (Srivastava et.al. 1999) and is considered as a stress indicator (Gould et.al. 1976). Aspartate amino transferase is the main transaminase that interferes with TCA cycle in a major way (Lowenstein 1967, Rao et al. 1984, Al-Attar 2010). A rise in its activity indicates the occurrence of greater energy demand which is normally associates with synthetic activities of the cell (Meister 1955). The intoxication of cadmium combine with an enzyme to form an enzyme inhibition complex, which react with various functional groups of the enzymes inhibit the enzyme activity of major metabolic site (Joseph et al. 2011). Serum level of Aspartate amino transferase become elevated may be due to hepatotoxicity of liver cells. These enzymes are biomarkers to know the intensity of liver damage.

Creatinine level is indicator of kidney function. In the present study, creatinine showed a significant increase in fish exposed to Cadmium. These may be due to

the action of heavy metal on glomeruli filtration rate (El-Bagori 2001; Abbass et al. 2002) and/or cadmium may cause pathological changes to the kidney resulting in dysfunction.

Exposure of cadmium induced a significant rise in blood triglyceride level in the fish. This may be due to decreased thyroid secretion (hypothyroidism) or liver dysfunction as the liver is the principal center of lipid metabolism.

The decreased activity of alkaline phosphatase indicates disturbances in the structure and integrity of cell organelles, like endoplasmic reticulum and membrane transport system. Depletion of enzymes in the fishes exposed to cadmium observed in the present study can be attributed to increased cadmium levels in the tissues. Furthermore accumulation of cadmium in liver and muscles could be the possible reason for variation of enzyme activities. The inhibition in protein level may also be due to the decrease in alkaline phosphatase activity as it plays an important role in protein synthesis (Pilo et. al., 1972).

The present study showed that cadmium altered the entire biochemical metabolism in *Oriochromis niloticus* by changing the levels of Total glucose, total protein and total cholesterol, AST, Alkaline Phosphatase, Creatinine and GPT in serum. Such changes in biochemical levels under the effect of cadmium toxicity might results in impairment of energy requiring vital processes, and hence give an idea about the health status of the fish population.

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